

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Lal *et al.*
Application No. 09/763,397

Art Unit: 1645

Filed: February 16, 2001

For: RECOMBINANT MULTIVALENT MALARIAL
VACCINE AGAINST PLASMODIUM
FALCIPARUM

Examiner: Vanessa L. Ford

Date: June 4, 2002

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service on June 4, 2002 as First Class Mail in an envelope addressed to: COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.

[Handwritten signature]
Tanya M. Harding, Ph.D.
Attorney for Applicant

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WASHINGTON, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.131

I, Ya Ping Shi, hereby declare as follows:

1. I am a co-inventor of the subject matter described and claimed by the patent application referenced above, *i.e.*, United States application No. 09/763,397 (hereafter the '397 application). I currently am employed by the Centers for Disease Control and Prevention (CDC), the assignee of the '397 application, which is located in Atlanta, Georgia. I was employed by the CDC while developing the invention described and claimed in the referenced application.

2. I understand that claims pending in the present application have been rejected in view of Gilbert *et al.*, *Nature Biotechnology*, 15: 1280-1284, 1997. I understand that Gilbert *et al.*, has been cited as allegedly anticipating certain claims pending in the referenced application, or, in the alternative, as allegedly rendering the claimed embodiments obvious.

3. The publication date of Gilbert *et al.*, is November 1997. United States Provisional Application No. 60/097,703 was filed on August 21, 1998. However, the co-inventors named on the '397 application invented the subject matter covered by the claims pending in the '397 application well prior to the November 1997 date that Gilbert *et al.*, became available as a reference.

4. Accompanying this Declaration as Exhibit A are photocopies of pages from my laboratory research notebook. These copies are true and accurate facsimile copies of photocopies

of the corresponding pages from my laboratory notebooks. All dates stated on these pages have been redacted.

5. All entries on the notebook pages of Exhibit A were made prior to November 1997.

6. The ideas and concepts demonstrated by Exhibit A arose from work conducted for the CDC in Atlanta, GA. These ideas and concepts are embodied in the claims of the '397 application. Thus, conception and reduction to practice of the invention recited in the claims of the '397 application, as discussed in more detail below, occurred in the United States of America prior to November 1997.

7. Exhibit A consists of 21 pages of laboratory notebook pages. Exhibit B consists of one page of CDC Biotechnology Core Facility Records. The contents of these pages of Exhibits A and B, and pertinent statements made on these pages are discussed below.

A. Exhibit B is a record from the CDC Biotechnology Core Facility showing the dates of my request for oligonucleotide synthesis, and the sequences of the requested oligonucleotides. These requests were made prior to November 1997. These oligonucleotides were used as is depicted in Figure 2 of the specification to amplify the synthetic vaccine antigen gene construct using Polymerase Chain Reactions (PCRs). The oligonucleotides of Exhibit B consist of both forward and reverse complementary sequences of SEQ ID NO: 1 of the application, with overlapping sequences acting as primers for the amplification in either the forward or reverse direction.

B. Pages 1-6 of Exhibit A display the planning strategy for the PCR synthesis of the synthetic gene construct. Set forth are relevant calculations for PCR reactions and primers used to generate quantities of the synthetic gene construct. Also shown are electrophoresis gels used to visually confirm the size of PCR-generated products.

1) Page 1 shows the calculation and strategy for serial PCRs. As is set forth at the top of page one, "AA" was my short hand for the PCR reaction involving oligonucleotides G0, GL, G1, and G2 of Exhibit B. "BB" was my short hand for the PCR reaction involving oligonucleotides G3-G6, and "CC" was my short hand for the PCR reaction involving oligonucleotides G7-G12.

2) Pages 2-5 show several experiments, ending with success as indicated by my comment "works well" on page 5. Reactions DD-II as depicted were successive rounds of PCR that joined the amplified fragments into the final synthetic gene construct.

C. Page 6 shows an electrophoresis gel on which I ran and visualized four samples at different concentrations from PCR reactions (the central four bands of the gel). Next to the gel is my comment "good!" indicating that the size of the band corresponding to the PCR product in each sample appeared to be the correct size.

D. Pages 7-10 shows that the PCR product was isolated and purified from the electrophoresis gel shown on page 6. Next, the purified product was cut with restriction endonucleases with BamH1 and Not1 (shown as steps #3 and #4 in Figure 2) for cloning into the expression vector pBluescript. The resulting sequence was SEQ ID NO: 1 of the application. Also shown on page 8 are the ligation reaction conditions for the ligation reaction, followed by restriction endonuclease reactions to evaluate the success of the ligation. The vector containing the fragment was then transformed into cells and plated onto agar plates. Positive clones were identified by their white color, indicating that the blue color-producing gene characteristic of a vector without a cloned segment had been interrupted with a cloned fragment. Page 10 sets forth the conditions for the PCR reaction to confirm that the correct gene fragment had been ligated into the vector (*i.e.*, to identify positive clones). My notation indicates that seventeen positive clones (numbers 1-4, 6, 8, 17, 21, 22, 25-27, 31, 33, 36, 39 and 40) were identified.

E. Page 11 shows an electrophoresis gel displaying samples of the PCR products.

F. Page 12 shows a single and double digesting experiment to confirm that the cloned fragment was properly oriented and was of the correct size. Clones 3, 26, and 33 were discarded by this experiment, leaving fourteen correct clones.

G. Page 13 shows the methods for the transformation of two plasmids, pBacPAK8 and pBacPAK9 with the synthetic gene construct for expression in Baculovirus. Also shown is an electrophoresis gel displaying samples of digested and undigested plasmid.

H. Page 14 shows another electrophoresis gel containing samples of DNA that were purified and digested with restriction endonucleases Not1 and BamH1, to confirm that the cloning into the Baculovirus expression vectors had been successful. My notation "orders are no problem" indicates that the clones were correctly oriented, and my statement "confirm 11, 20, 63 clones are true clones" indicates that I considered these clones to be successful. The depicted gel

shows the results of restriction endonuclease digestion showing the two bands of each clone (lanes 4, 5 and 8, respectively). I identified clone number 20 as the clone that would be sequenced to confirm correctness at the molecular level. The sequencing indicated that clone 20 contained a single mutation. Therefore, I sequenced a second clone, number 63. The sequencing indicated that clone 63 also contained a single mutation. In comparing the location of the mutations in clones 20 and 63, I found the mutations were located in different segments. Thus, I subsequently performed experiments to generate a subclone that would contain the correct segments of clones 20 and 63.

I. Page 15 sets forth conditions for methylation experiments, which were run to protect restriction endonuclease sites in the vector. Following these reactions, the correct segments of clones 20 and 63 (as shown on the bottom of pg. 15) were excised and ligated into the Baculovirus expression plasmid pBacPAK8.

J. Page 16 shows a basic diagram of the recombinant vaccine antigen gene as cloned into the Baculovirus expression vectors. As shown, the construct contains portions of both clones 20 and 63.

K. Page 17 shows a gel wherein the products of the second ligation reaction of the correct segments of constructs 20 and 63 into the pBacPAK8 expression vector were run to confirm the size of the construct.

L. Page 18 shows the success of my ligation experiment, as confirmed by visualization of the bands on the electrophoresis gel. My statement that "clones 21, 31, 33, 35 are positive" indicates that the ligation reaction was successful.

M. Page 19 shows an electrophoresis gel displaying the results of BamH1 restriction endonuclease digestion, to confirm the successful clones.

N. Page 20 shows an electrophoresis gel displaying the results of BamH1 and NotI restriction endonuclease digestion. My notation "save clone 31A and 31B" indicates that these clones were successful. I subsequently confirmed these results by sequencing clones 31A and 31B.

O. Page 21 contains my notation "miniprep for sending product to Hessian (sic)." This refers to co-inventor Dr. Seyed Hasnain, who tested the expression of the synthetic gene construct in the Baculovirus expression system.

8. All statements made herein and of my own knowledge are true and all statements made on information are believed to be true. Furthermore, these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements made may jeopardize the validity of the application or any patent issuing thereon.

26/05/2002

Date

Ya Ping Shi

Name Ya Ping Shi

EXHIBIT A

First PCR

| | | |
|--------------------|-----------|---|
| A.A: G1 - G2 | 1 μl | 94°C 5min |
| B.B. G3 - G6 | 5 μl 50°C | 94°C 45" |
| C.C. G7 - G12 | 1 μl 65°C | 45°C 1 min 72°C 1.5 min 8 cycle (P139) |
| String buffer | | |
| AA: 2 x 4 = 8 μl | 73.5 | 16 μl dNTP |
| B, B 2 x 4 = 8 μl | 65.5 | 1 μl Buffer |
| | | 0.5 μl Taq |
| | | <hr/> 26.5 μl. |
| C.C. 2 x 6 = 12 μl | 61.5 | |

| | | |
|------------|---|--|
| Second PCR | 16 μl dNTP 1 μl Buffer 0.5 μl Taq | 94°C 5min. 94°C 45" 45°C 1 min 72°C 1.5 min |
| | 5 μl Oligo 1 5 μl Oligo 2 | 25 cycle (P141) |

| | | | |
|------------------------|--------|--------------|------------|
| DD ₁ | 1 μl | 52.5 + 5 + 5 | <hr/> 46.5 |
| AA/ DD ₂ | 2.5 μl | 51 + 5 + 5 → | G0 |
| DD ₃ | 5 μl | 48.5 + 5 + 5 | G2 ✓ |
| DD ₄ | 10 μl | 43.5 + 5 + 5 | |
| GB ₁ | 1 μl | 52.5 + 5 | |
| GB ₂ | 2.5 μl | 51 + 5 → | G3 ~ |
| GB ₃ | 5 μl | 48.5 + 5 → | G6 ~ |
| GB ₄ | 10 μl | 43.5 + 5 | |

C { FF₁ 1 μl 52.5 + 5
FF₂ 2.5 μl 51 + 5 → G7 ✓
FF₃ 5 μl 48.5 + 5 G12
FF₄ 10 μl 43.5 + 5

Re do CC₀: G₇ - G₁₂ = 12 uL.

| | | |
|------------------|----------------|---------|
| dNTP | 16 uL | c.; Taq |
| 10x Buffer | 10 uL | |
| H ₂ O | <u>61.5 uL</u> | |
| | 100 uL | |

94°C 5 min

94°C 45"

40°C 1 min

72°C 2 min

8 cycle.

CC₁ G₇ - G₈ (only do second PCR) = 4 uL + 69.5 uL
 CC₂ G₉ - G₁₂ 2 x 4 = 8 uL + H₂O · 65.5 -

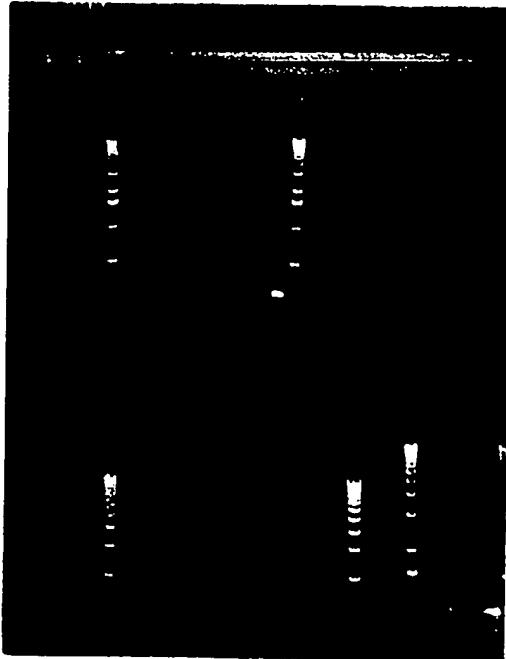
Do SOE G₆ - G₇

| | | |
|-----------------------------------|------------------------|--------------------------|
| DD ₁ + EF ₁ | H ₂ O | 16 uL dNTP |
| G _G ₁ | 1 uL + 1 uL = 2 uL | 61.5 63.5 10 uL Buffer |
| G _G ₂ | 2.5 uL + 2.1 uL = 5 uL | 58.5 5 uL G ₆ |
| G _G ₃ | 5 uL + 5 uL = 10 uL | 53.5 5 uL G ₆ |
| G _G ₄ | 10 uL + 10 uL = 20 uL | 43.4 0.5 Taq |
| | | <u>36.5</u> |

program 141

| | | | | |
|-----------------|--|--------|-----------------------|--------------|
| FF ₁ | primers G ₇ G ₁₂ 10 uL | 63.5 | H ₂ O | 16 uL dNTP |
| FF ₂ | | 1 uL | 62.5 | 1 uL Buffer |
| FF ₃ | | 2.5 uL | 61 | 0.5 uL 10 uL |
| FF ₄ | | 5 uL | 58.5 | Taq 0.5 |
| FF ₅ | 10 uL | 53.5 | | |
| FF ₆ | .. | 1 uL | 94°C 5 min - 36.5° | |
| FF ₇ | .. | 2.5 uL | 94°C 45" | |
| FF ₈ | .. | 5 uL | 40°C 1 min .. | |
| | .. | 10 uL | 72°C 2 min | |
| | | | cycles #41 | |

Result G₇G₁ - 4



i-F₁-3 did not work probably because oligo?

Prepare new temp oligo G₉ - G₁₂. also A₁-1065.

Redo: $CC_2' \rightarrow CC_2''$ and CC_3'' .

→ 3.5

CC_2'' G₉ G₁₀ G₁₁ G₁₂ × 2 = 8ul . 65.5

CC_3'' G₉ G₁₀ G₁₁ A11064 × 2 = 8ul . 65.5

X
works well

16ul dNTP.

10ul Buffer

0.5 Tag

Same to before:

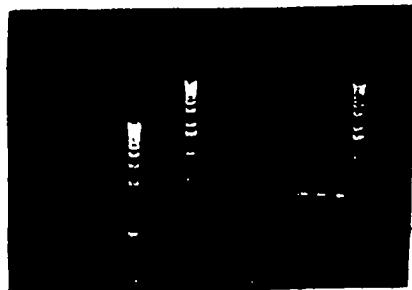
Second PCR.

| | | | | | |
|-----------------|-------------------|---------|-------|------|------------|
| FF ₁ | CC ₂ ' | primers | 1ul | 1ul | 16ul dNTP |
| FF ₂ | | | 2.5ul | 6.1 | 1ul buffer |
| FF ₃ | | | 5ul | 58.5 | 0.180 10ul |
| FF ₄ | | | 10ul | 53.5 | Tag 0.5ul. |

| | | | | | |
|-----------------|-------------------|--|-------|------|------------|
| FF ₅ | CC ₃ ' | G ₉ A11064 works well | 1ul | 1ul | 16ul dNTP |
| FF ₆ | | | 2.5ul | 6.1 | 1ul buffer |
| FF ₇ | | | 5ul | 58.5 | 0.180 10ul |
| FF ₈ | | | 10ul | 53.5 | Tag 0.5ul. |

Same to before.

1141





714

II



5

SoE for G₇ - G₁₁ + AL-1065.

| | CC' | FF" | H ₂ O | 63.5 | 1614 dNTP |
|----------------|--------|----------|------------------|------|---------------------|
| H ₁ | 1 ml | + 1 ml | 61.5 | | 10.00 buffer |
| H ₂ | 2.5 ml | + 2.5 ml | 58.5 | | 5 ml G ₇ |
| H ₃ | 5 ml | + 5 ml | 53.5 | | 5 ml AL-1065 |
| H ₄ | 10 ml | + 10 ml | 43.5 | | 0.5 TAA |
| | | | | | <u>36.5</u> |

Program II 41

| | G _{G7} | + H ₁ | H ₂ O | 63.5 | 1614 dNTP |
|----------------|-----------------|------------------|------------------|------|--------------|
| I ₁ | 1 ml | + 1 ml | 61.5 | | 10.00 buffer |
| I ₂ | 2.5 ml | + 2.5 ml | 58.5 | | 5 ml AL-1064 |
| I ₃ | 5 ml | + 5 ml | 53.5 | | 5 ml AL-1065 |
| I ₄ | 10 ml | + 10 ml | 43.5 | | 0.5 TAA |
| | | | | | <u>36.5</u> |

Program II 41

good!

Further cleaning and cloning,
sequencing -

A: Run gel and cut ~~out~~ and clean.

- ① Gene clean (from product of PCR)
- ② gel clean through column (according instruction of manufacturer) (Soak off PCR product tube (one is pellet (store in -20°C))
and add ~~water~~ 20 μl water. From this, 10 μl was take for digestion.

B: digestion:

Not I : 26 μl Water
3 μl Buffer
1 μl Not I (I.I. Gene clean
II. Column clean)
 ↓
1 h 37°C pellet.

BamHI : 26 μl H₂O
3 μl buffer
1 μl BamHI
— 1 h 37°C

Ligation

| | | | |
|--------------|-------|--------------------------|------------|
| Water | 13 μl | Control I | Control II |
| Vector | 1 μl | (BamHI and Not I digest) | .. |
| 5×lig buffer | 4 μl | .. | 15 μl |
| T4 ligase | 2 μl | .. | x |

overnight (4°C)

Not I digestion:

vector:

| | |
|------------------|-------------------------|
| 1 μl | vector (concn 3.2ug/μl) |
| 3 μl | 10x buffer |
| 3 μl | BSA |
| 4 μl | Not I |
| 10 μl | <u>H₂O</u> |
| 30 μl 37°C 1.5 h | |

target

II₂ and control (MSP-1)

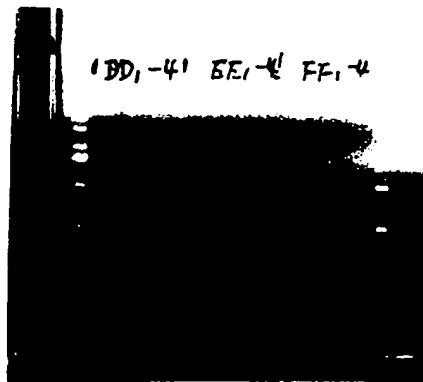
| |
|------------------------------|
| 2 μl H ₂ O |
| 3 μl BSA |
| 3 μl 10x Buffer |
| 2 μl <u>E₂914</u> |
| 30 μl 37°C 1.5 h |

~~Not~~ BamH I digestion

| | | |
|--------|--------|--------------|
| vector | BamH I | 4 μl |
| | Buffer | 3 μl |
| | water | <u>2.3</u> |
| | | <u>30 μl</u> |
| | | 37°C 1.5 h |

| | |
|--------|--------------|
| vector | 2 μl |
| Buffer | 3 μl |
| water | <u>25 μl</u> |
| | <u>30 μl</u> |
| | 37°C 1.5 h |

Result



FF₁-4 did not work because first PCR (CC) annealing temp was too high.

Need redo CC (first PCR), then FF₁-FF₄

Ligation (2) before
transformation as before

result. not so much white clones. probably vector
was not properly digested.
Clump further purify vector.

pick up 40 clone grow overnight
cell PCR: as regular. 10ul cell 94°C 5 min.

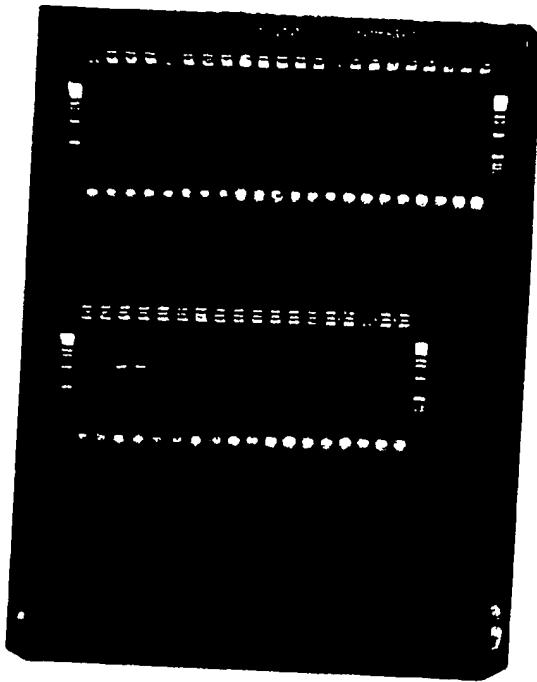
| | | |
|-------|--------|----------------------|
| ELIGO | AL1064 | 2.5ul |
| | AB1065 | 2.5ul |
| | Buffer | 5ul |
| | dNTP | 8ul |
| | Taq | 0.5 |
| | 1+20 | <u>21.75</u> 40ul |

15 cycle 94°C 45" 50°C 45" 72°C 60"

positive clone

1, 2, 3, 4, 6, 8, 17, 21, 22,
25, 26, 27, 31, 33, 36, 39, 40,

mix each

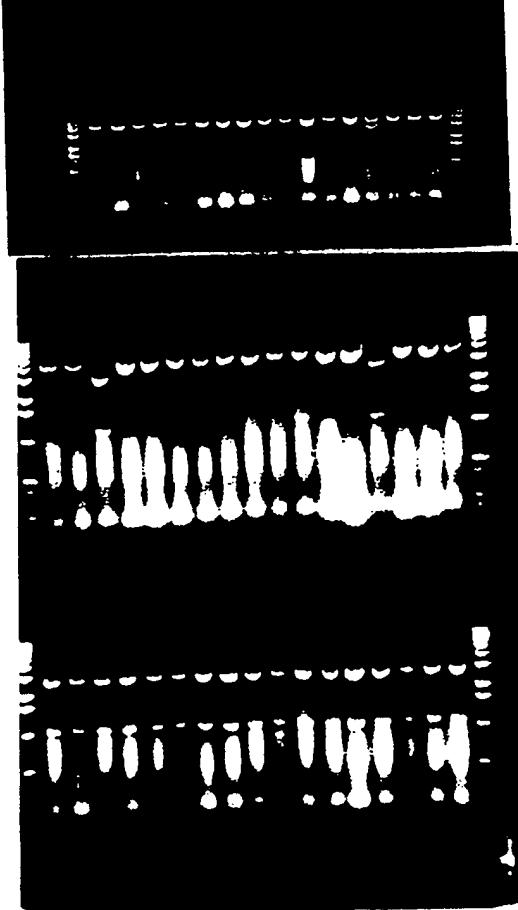


Save Seed
Purified Squalene Vaccine
Salinas

c) digest all positive (17) clones (based on PCR)

Single digestion: BamHI or NotI
double digestion BamHI and NotI

Result: Clone 3, 26, 33 are not pure clones.
discard or don't use them



6
7

Plasmid pBacPAK8 and pBacPAK9 (from Sanger)
2¹⁴g / 100ul 2¹⁴g / 100ul

Transformation:

100ul plasmide (200ng)

100ul XL-blue cell

procedure as regular.

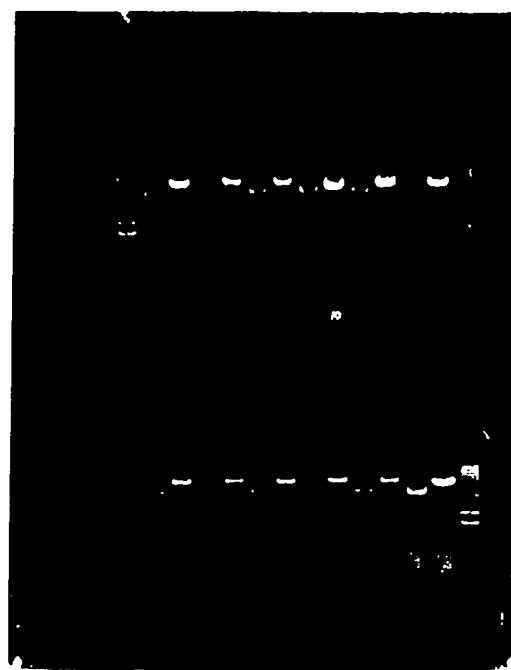
plating overnight
growth well

Mini prep of pBacPAK8 and pBacPAK9 -

run undigested and digested plasmid

100ng / uL $\times 19 =$

1.9uL



13

digested

This result confirm that ~~two~~ orders are no problem. also confirm (11) (20) (63) clones are true clones:



I will sequence clone 20.

Methylation:

Clone 63 Vector correct.

Clone 20 Mest target correct

Clone 63 methylation.

Reaction: 3ul Tagi methylase
 3ul NEB 4 Buffer
 0.3ul BSA
 2.2.2ul H₂O
 1.5ul Mix SAM

1 hr 65°C

Mix: 5oul NEB 4 Buffer + 4.5oul H₂O + 1.25ul SAM

6.6ul NaCl (5M)
6oul Ethanol (100%)

Hind II cut

clone 63 (two pieces very big)

clone 20 (more pieces vector small)

run gel standard

20 63 standard

(unresolved) (+ve big)

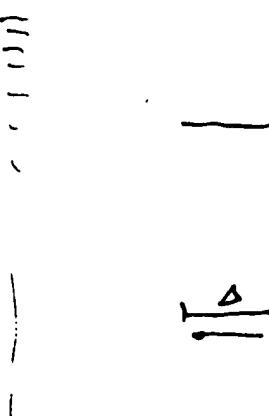
reaction conditions:

3 μl buffer
6 μl HindII
21 μl H₂O
1.5 h 37°C

Result:

clone 20

clone 63

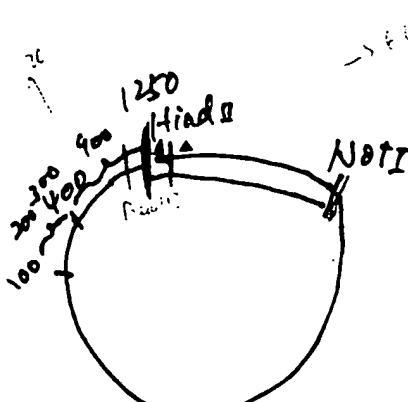


1 kb

1 kb
0.9 kb

— 0.9 kb

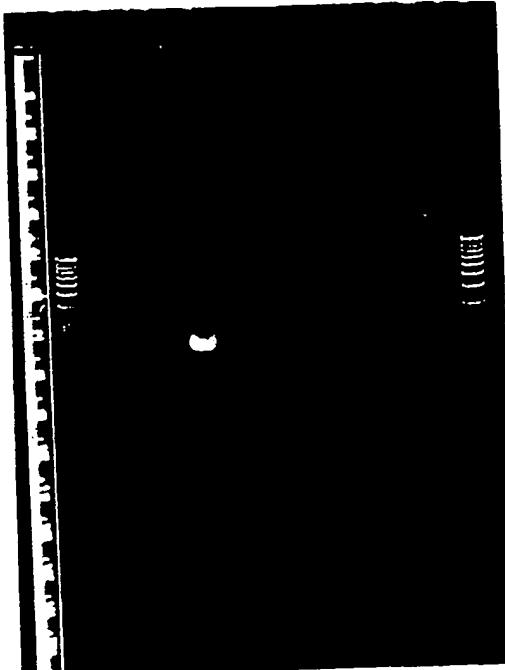
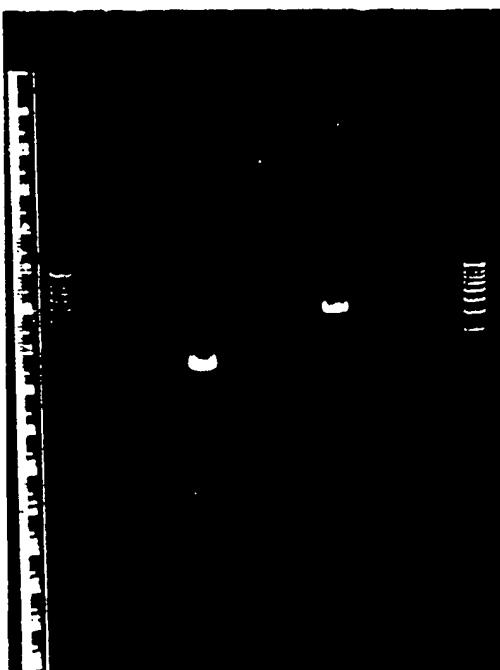
— — 1 kb



△ cut fragment

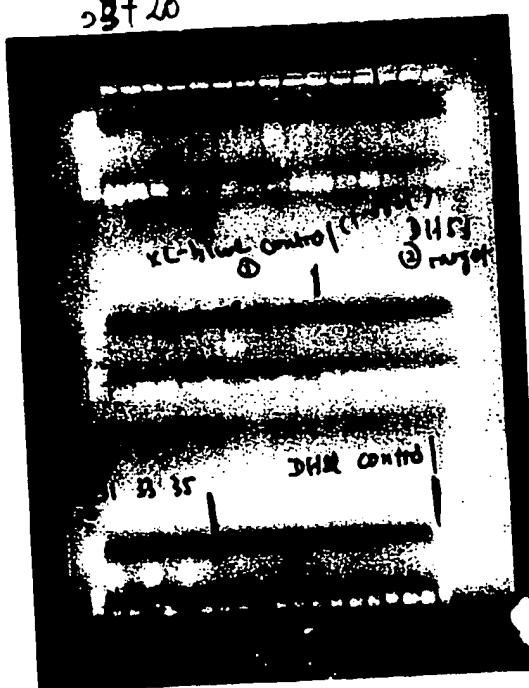
16

Ligation: as routine



clone 63 + 20 ligation see before

clone PCR Dilution: AL1097
AL1064



lanes 21, 31, 33, 35
are positive.

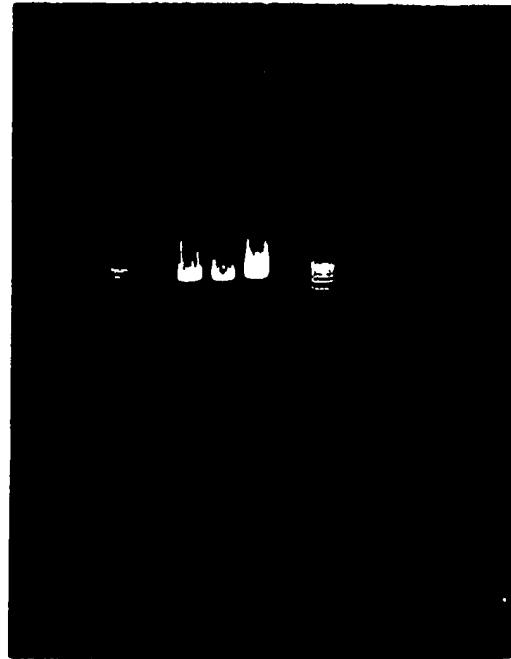
Save as Name:

~~38+20/ct-1721/63+20~~

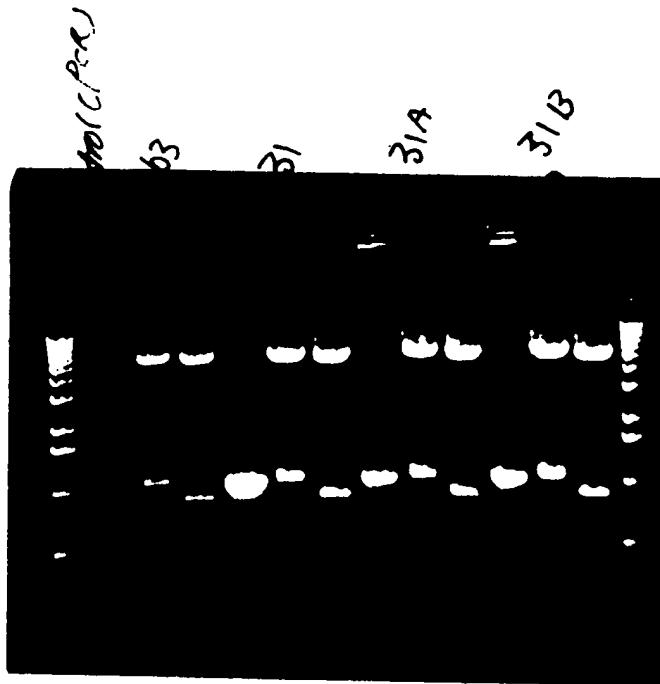
number

PacB/63+20/number

BamHI digestion: 63+20 (21, 31, 33, 35) 6:



original bacterial methylation



Save clone 31A and 31B
named as pac8/63^{r20}/31A and 31B

EXHIBIT B

ANSWER

Page 1

| Date | Oligo ID | DNA Syn No. | Sequence | Length-mer |
|------|----------|---|----------|------------|
| G0 | 953882 | GTCGGGATCCATGAAATTCTTAGTCACCGTGCCTTGTT | 94 | |
| GL | 953883 | TTATGGTCGTACATTCTTACATCTATGCCGGalcatca tcatcatcat ttatcttcacatcacatcgatcgatcgatcgatcgat | 40 | |
| G1 | 953884 | gaaggtaaagatgaagataaaAGAGATGGAATAACGAAGAAC GAGAAATTAGGAAACCAAAACATAAAAATTAAAGCAAC CAGGGGATGGTAATCCTtgcctcgatcgatcgatcgat | 120 | |
| G2 | 953885 | tattacgacatcaaaccacATGGAACATTTCATTTACAATT TTTTTCAATATCATTTCTAATCTAATTCTGCTTGGTT TTCCACAAAGTtacactacatgggaccaag | 120 | |
| G3 | 953886 | TGTGTTAATGTCGTAATAGTAATTCTGGATGTTCAGA CATTAGATGAAAGAGAAGAATGTAATGTTATTAGAAG ATTCAAGGTAGCAACGGAAAGAAATCACATGTAATGTC ttgggtttgcatttcacatcgatcgatcgatcgatcgat | 120 | |
| G4 | 953887 | tggtatatttacaatTCATCAGGATTGCAATTGGGTTGCGT AGAATCAGGTTAgtatccatcgatcgatcgatcgatcgat ttgggtttgcatttcacatcgatcgatcgatcgatcgat | 120 | |
| G5 | 953888 | CTGGAAATGCTGAAATTGTCATTGGGTTGCGT AACATTAGGAAAtactccactccatttagaag gcatgttgtatgaatgttGCTTAGTCAGTTACCTATAATTAG AATTCGCTTTATATAGTGTACAAACTTTTACATTGTT TATATAATTcttataatggatggat | 120 | |
| G6 | 953889 | AAGATATTGTTGAAagatttatataatgata aaccatcaaaacaatgtGGACAACATGGACATATGCATGGTA ACGGAGGGAGGATGGAGGAACGCTACTAAGGAATATG cttaatttttataatctgttAAATGTTGTCATATTGTTAGGTTGA TCACCTTTGATATGGATGTTCCCAATAATTCTGTCCTCT TCCAAAGCTtatccatataatgtaaactt | 120 | |
| G7 | 953890 | agatataggaaaaatataagAAGGTAAGGCCCTGGATAATTGGA AATATCTATGATTATCACTATGAGCATTCTAGTCATCTAG TACAAAGTCATCAagtccatcaaatgtaaaatc atccatatattcgatcaaCCTTTCAATAACTTACCGAAGAA ATCTCTGATTTCAGCTTTAATTTCATTAACTCTTGTAG CTAGACTAgatgtatcgatcgatcgatcgatcgatcgat | 120 | |
| G8 | 953891 | tttagcgaaataagatGATTAGAAGTTAGTAGTACAGGAGATA TGTCCGCAAGAATCAAATAGATATAACTATTCTTATTgaa tagggggccgcgcac gtcgccgcgcgcac | 104 | |
| G9 | 953892 | tttagcgaaataagatGATTAGAAGTTAGTAGTACAGGAGATA TGTCCGCAAGAATCAAATAGATATAACTATTCTTATTgaa tagggggccgcgcac gtcgccgcgcgcac | 104 | |
| G10 | 953893 | tttagcgaaataagatGATTAGAAGTTAGTAGTACAGGAGATA TGTCCGCAAGAATCAAATAGATATAACTATTCTTATTgaa tagggggccgcgcac gtcgccgcgcgcac | 104 | |
| G11 | 953894 | tttagcgaaataagatGATTAGAAGTTAGTAGTACAGGAGATA TGTCCGCAAGAATCAAATAGATATAACTATTCTTATTgaa tagggggccgcgcac gtcgccgcgcgcac | 104 | |
| G12 | 953895 | tttagcgaaataagatGATTAGAAGTTAGTAGTACAGGAGATA TGTCCGCAAGAATCAAATAGATATAACTATTCTTATTgaa tagggggccgcgcac gtcgccgcgcgcac | 104 | |
| | | | 20 | |